



Faculty of Resource Science and Technology

**THE EFFECT OF TEMPERATURE ON FERULIC ACID
PRODUCTION BY *Aspergillus fumigatus* (F01) FROM
SAGO BARK FOR GENERATING BIOVANILLIN**

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(F01) from Sago Bark for Generating Biovanillin**

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This project is submitted in partial fulfilment of the requirement of the degree of
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DECLARATION

I hereby declare that this Final Year Project report 2015 entitled “**The Effect of Temperature on Ferulic Acid Production by *Aspergillus fumigatus*(F01) from Sago Bark for Generating Biovanillin**” is based on my original work except for the quotations and citations which have been dully acknowledged. It has been or concurrently submitted for any other degree at UNIMAS or other institutions of higher learning.



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LIST OF ABBREVIATIONS

| | |
|-------|-----------------------------|
| DSRH | Destrached rice husk |
| FA | Ferulic acid |
| FAe | Ferulic acid esterase |
| Mg/g | Miligram per gram |
| Mg/ml | Miligarm per mililiter |
| MSM | Minimal salt medium |
| PDA | Potato Dextrose Agar |
| rpm | Revolutions per minute |
| SSF | Solid State Fermentation |
| U/g | Unit (U) per gram of enzyme |

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The Effect of Temperature on Ferulic Acid Production by *Aspergillus Fumigatus* (F01) from Sago Bark for Generating Biovanillin

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ABSTRACT

Vanilla (4-Hydroxy-3-methoxybenzaldehyde) most commonly used as food flavouring agents. The demand for natural vanillin especially the amount of natural vanillin available that extracted from the vanillin pods is high. This has led to the investigation of alternative ways for the production of this flavour such as the biotechnological production from microorganisms known as biovanillin. Biovanillin can be produced by using different sources of microorganism including *Aspergillus fumigatus*. To produce biovanillin, ferulic acid (FA) was used as vanillin precursor. Therefore, the aim of the study is to produce FA from *A. fumigatus*. This was done via solid state fermentation (SSF) by using sago bark as substrate. Different range of temperature (30°C-40°C) that effects the FA production was studied. From this experiment it is found that the optimal temperature to produce high FA production was at 35°C with duration of 10 days of incubation time. The amount of FA produced was 0.8812 mg/g with 0.2643 U/g amount of ferulic acid esterase (FAe) activity.

Key words: Solid state fermentation (SSF), ferulic acid (FA), ferulic acid esterase (FAe), sago bark, and *Aspergillus fumigatus* (F01).

ABSTRAK

Vanilla (4-Hydroxy-3-methoxybenzaldehyde) adalah agen perisa dalam makanan yang sering digunakan. Permintaan untuk vanilla semula jadi yang diekstrak daripada pokok vanilla adalah sangat tinggi. Oleh itu, terdapat pelbagai cara alternatif yang boleh digunakan, dikaji untuk menghasilkan vanilla seperti pengeluaran bioteknologi dari mikroorganisma yang dikenali sebagai biovanillin. Biovanillin boleh dihasilkan daripada bermacam jenis mikroorganisma yang berbeza termasuklah *Aspergillus fumigatus*. Penghasilan biovanillin memerlukan asid ferulik sebagai peransang vanilla. Justeru, tujuan kajian ini adalah untuk menghasilkan asid ferulik dari *A. fumigatus*. Kajian ini dijalankan melalui fermentasi keadaan pepejal dengan menggunakan kulit pokok sago sebagai substrat. Suhu yang mempengaruhi pengeluaran asid ferulik telah dikaji. Hasil kajian mendapati bahawa suhu optimum yang diperlukan untuk menghasilkan jumlah asid ferulik yang tinggi adalah pada 35°C dalam tempoh 10 hari. Jumlah asid ferulik yang dihasilkan adalah sebanyak 0.8812 mg/g dengan jumlah aktiviti asid ferulik esterase sebanyak 0.2643 U/g.

Kata kunci: Fermentasi keadaan pepejal, asid ferulik, asid ferulik esterase kulit pokok sago, dan *Aspergillus fumigatus* (F01)

1.0 INTRODUCTION

Sago palms or also known as *Metroxylon sagu* is mainly grown in the area outside the Peninsula which is in the state of Sarawak. According to Malaysia Agriculture Economics Association, Sarawak is the biggest exporter of sago and about 50 to 110 ton of sago wastes are produced daily (Singhal *et al.*, 2008). The by-product of sago consist of basically stem, palm and leaves. Sago waste such as sago bark, sago hampas, and the leaves can be used for the by-products production. However, the accumulation of sago waste has been a concern due to improper treatment and disposal of this waste. Sago wastes are typically disposed by combustion, which brings detrimental effect not only to environment but also to human being.

Thus, research had discovered that some agriculture by-product including this sago waste can be exploited for various bioconversion processes. For instance, the waste from sago has been used as the substrate for kojic acid production, ethanol for fuel, and production of fermented sugar (Singhal *et al.*, 2008). Among other bio-product that can be potentially produced from the sago waste is biovaillin. Biovanillin is a new alternative substance that was discovered to produce the same flavouring component as the natural vanilla.

Vanilla is one of the world most popular flavouring and aromatic component that is extracted from the vanilla orchid pods of *Vanillin planifolia*. However, according to Converti *et al.* (2010), due to its high cost and less of the natural product in the market, only 1 % of the worldwide production of vanillin comes from natural vanilla. Thus, the chemical synthesis of vanillin from guaiacol and glyoxylic acid was developed to overcome this problem (Silva *et al.*, 2009). It is an artificially vanilla flavor derives from

the chemical process. Unfortunately, US and European legislation not classified this synthetic vanillin as a natural product as reported by Muheim and Lerch (1999).

On the other hands, biovanillin has the potential to become economically competitive in the future. This is because according to Zamzuri and Abd-Aziz (2013), biovanillin is produced biologically by microorganisms from a natural precursor that can produce same flavouring agent as natural vanilla. As mentioned by Converti *et al.* (2010), to make the process economically viable, it is necessary to find a precursor chemically close to vanillin which is less cost and easily to get.

Fortunately, this agriculture waste containing ferulic acid (FA). FA is phenolic acid in the plant that has many functions such as anti-oxidation, antimicrobial and anticancer activities (Zhang *et al.*, 2012). While in food industry, this FA is used as the ingredient component. In addition, recovery of FA from agriculture waste is importance as it is a precursor for vanillin production (Marcia *et al.*, 2012). This FA is released by using polysaccharide degrading enzyme and specific ferulic acid esterase (FAe).

The FA is converted by a two step process using first *Aspergillus fumigatus* to transform the FA from the lignocellulosic component into vanillic acid. Then, *Phanerochaete chrysosporium* will be responsible for vanillic acid modification into vanillin. Moreover, this bioconversion process is done through the solid state fermentation process (SSF).

Thus, the focus of this study is to employing the application of SSF for production of desire product with the present of microorganism as bioconversion agent. Specifically the objectives of this study are:

- i) To identify the optimal temperature for FA production through SSF by *A. fumigatus*.
- ii) To determine the effect of temperature on FA generation from sago waste by *A. fumigtus* for generating biovanillin.
- iii) To determine the ferulic acid estrerase activity from SSF by *A.fumigatus* at different temperature.

2.0 LITERATURE REVIEW

2.1 Sago palm and it waste composition

In Malaysia, sago palms or also known as *Metroxylon sagu* is mainly grown in the area outside the Peninsula which is in the state of Sarawak. According to Malaysia Agriculture Economics Association, Sarawak is the biggest exporter of sago and currently, sago export ranks as the fourth biggest agricultural income for Sarawak, after oil palm, pepper, and cocoa. Besides that, Sarawak also exporting annually about 25,000 to 40,000 tons of sago products to Peninsular Malaysia, Japan, Taiwan, Singapore, and other countries (Chew, *et al.*, n.d.).

In Sarawak, about 50 to 110 ton of sago wastes are produced daily (Singhal *et al.*, 2008). Sago waste is a starchy lignocellulosic by-product generated from pith of the sago palm after starch extraction (Sun & Cheng, 2002). This lignocellulosic composed of three groups of polymer which are cellulose, hemicellulose and lignin.

Research shows that sago wastes contains approximately 66% starch and 14% fibre on a dry weight basis of which about 25% is made up of lignin (Zamzuri & Abd Aziz, 2013). Besides that, the dried sago waste contains about 60-70% starch as shown in **Table 2.1**.

Table 2.1: Composition of sago waste (Zamzuri & Abd Aziz, 2013)

| Component | % |
|---------------|------|
| Starch | 65.7 |
| Crude fiber | 14.8 |
| Crude protein | 1 |
| Fat | n.d. |
| Ash | 4.1 |
| Moisture | 59.1 |

Agriculture waste including sago waste can be potentially used for various bioconversion process due to amount of its beneficial residual compound such as starch, sugar and other nutrients (Chew, *et al.*, n.d.). As stated by Sun and Cheng (2002), sago wastes is inexpensive and may be used as animal feeds, compost for mushroom culture, for hydrolysis to confectioner syrup, and for particleboard manufacture. While, Singhal *et al.* (2008), noted that the utilisation of sago wastes as a cheap substrate that has great potential for agricultural by-product production through SSF.

2.2 Ferulic acid (FA)

Ferulic acid (FA) is a yellowish powder that belong to the family of hydroxycinnamic. As stimulated by Hartley and Harris (1981), FA is abundantly present in plant cell walls that link covalently to lignin or other polymers. FA is one of the most promising sources of antioxidant. Like many other antioxidants, FA reduces the level of cholesterol and triglyceride, thereby reducing the risk of heart disease. Moreover, FA seems to reduce the risk of many cancers, including cancer of the stomach, colon, breast, prostate, liver, lung and tongue.

The applications of FA and ferulic acid esterase (FAe) enzymes are many and varied. For instance, FA obtained from agricultural by-products is a potential precursor of vanillin for the production of natural biovanillin. Microbial or enzymatic transformation paths for biovanillin production can be achieved by using potential precursors like FA (Ramesh *et al.*, 2007). In order to produce vanillin, it is important to release FA from raw materials by enzymatic treatment and extraction, which later can be treated using various microorganisms (Ou *et al.*, 2007).

Ferulic acid esterase (FAe) have the ability to release FA from the agro industrial by product such as coffee pulp, wheat straw and apple marc. There is a growing interest in FAe as promising biocatalysts in the processing of hemicelluloses (Saha, 2003) pulp bleaching as well as in the production of phenolic acids such as ferulic, p-coumaric, caffeic, and sinapic acids as fine chemicals from natural sources (Faulds *et al.*, 1997). Moreover, the microbial transformation of FA is recognized as the most attractive and promising alternative source of natural vanillin. For example, a *Jatropha curcas* stem hydrolysate containing 1.55 g/L of FA, which was successfully used as substrate for one-step vanillin production by *Aspergillus niger* and *Pycnoporus cinnabarinus* (Vaithanomsat & Apiwatanapiwat, 2009).

2.3 Biovanillin as an alternative food flavour

Vanillin is a plant secondary metabolite and the main constituent of natural vanillin (Zamzuri & Abd-Aziz, 2013). Vanillin was first synthesized from eugenol in 1874, less than 20 years after it was first identified and isolated from clove oil (Hocking, 1997). Research shows that commercial vanillin available in the market can be divided into two types which are natural vanillin and synthetic vanillin (Stentelaire *et al.*, 2000). However, according to Li and Rosazza (2000), vanillin is divided into three types which are natural vanillin, synthetic vanillin and biovanillin.

Natural vanillin is the vanillin that obtained from pods of the *Vanilla planifolia* which mainly grown by small farmers in Madagascar, Mexico and across the Southeast Asia (Converti *et al.*, 2010). This natural vanillin has a very high and variable price in the flavour industry market. However, the high demand of this natural vanillin was limited due to the availability of vanilla pods. As reported by Silva *et al.* (2009), the unstable price of

natural vanillin and low supply in the market have changed development towards the chemical synthesis of vanillin. It is an artificially derived vanilla flavour from the chemical process. However, Krings and Berger (1998) reported that this synthetic vanillin was not regarded as a natural food component by European countries and USA.

Several researched have been proposed in order to produce vanillin through the biotechnological pathway to replace natural vanillin extracted from vanilla pods. Thus, in 1997, Pak *et al.* (2004) stated that the H&F Florasynth began the production of a vanillin identical to the natural one through a process that consists of two steps. The two-step process was described involving the transformation of FA into vanillic acid using *Aspergillus* sp. and later converted into biovanillin by *Pycnoporus cinnabarinus* or *Phanerochaete chrysosporium* which commonly used to degrade the lignin (Stentelaire *et al.*, 2000).

Lignocellulose is mainly composed of three groups of polymers which are cellulose, hemicellulose and lignin. In the production of biovanillin, lignin is important to produce FA that act as a precursor of vanillin. Biological treatments based on the use of brown, white and soft rot fungi have been commonly used to degrade the lignin (Mussatto & Teixeira, 2010). Degradation of lignin by fungi such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Trametes hirsuta* and *Bjerkandera adusta*, may be used to give better contact to the cellulose and hemicellulose components, also to be considered as an effective biological detoxification alternative (Mussatto & Teixeira, 2010).

There are several methods have been developed in order to enable vanillin and furanone or pyranone derivatives of natural origin to be produced from agricultural wastes which included the FA, (Figure 2.3). The basic process combines enzyme degradation of plant cell wall and fungal fermentation.

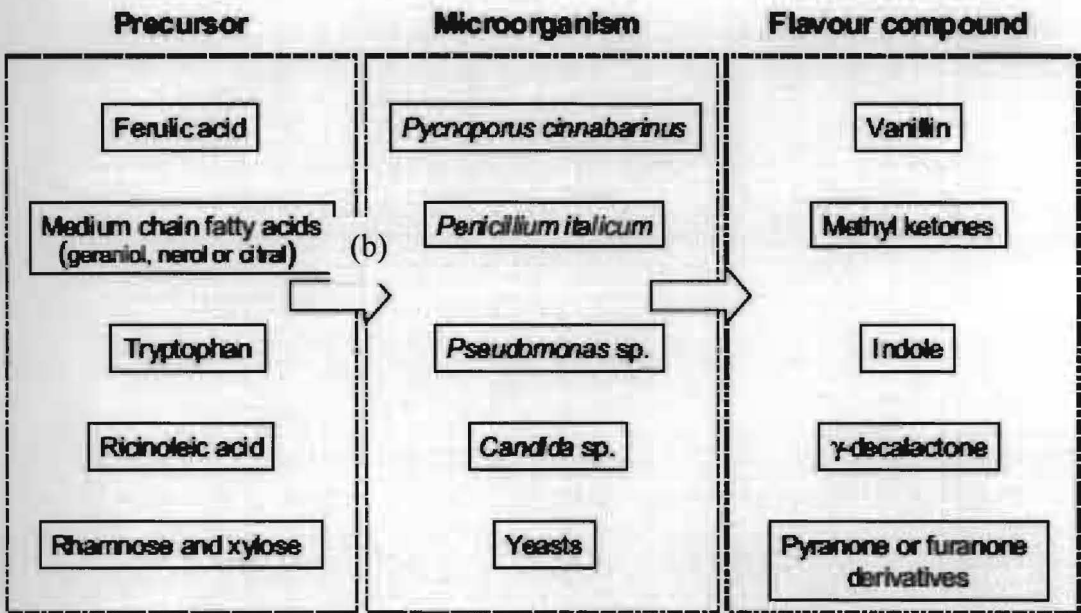


Figure 2.3: Some of the flavours produced by microbial bioconversion of the precursors including vanillin

2.4 Solid State Fermentation (SSF)

SSF is the cultivation of microorganisms under controlled condition with the absence of free water (El-Mansi *et al.*, 2007). Bhargav *et al.* (2008) mentioned that the aim of SSF is to bring cultivated fungi or bacteria in tight contact with the insoluble substrate and to achieve the highest nutrient concentration from the substrate for fermentation. Besides that, SSF is frequently applied in agro-waste bioprocess industry due to the close resemblance of the method to the natural condition of microbial cultivation.

The unique characteristic of SSF is that it can operate at low moisture level, which provides a selective environment for the growth of the mycelial microorganism such as fungi. Since bacteria and yeast cannot tolerate low moisture levels, the chances of contamination of fermentation media by bacteria or yeast are highly reduced in SSF (Shuler & Kargi, 1992). **Table 2.4** shows differences between solid state and submerged liquid fermentation (Raimbault, 1998).

Different agro industrial waste can be used as solid substrates. For example, palm biomass and rice bran (Zamzuri & Abd-Aziz, 2013) cassava bagasse and sugar cane bagasse (Bhargav *et al.*, 2008). The application of modern biotechnical knowledge and process control technologies may lead to significant increase of productivity from this ancient process.

However, during the SSF process there are some of the factors affecting the growth of the product such as temperature, pH, and moisture content. Thus, all this factors need to be maintained optimally during the SSF process to produce high desire product (El-Mansi *et al.*, 2007).

Table 2.4: Differences between Solid state and Submerged liquid fermentation (Raimbault, 1998)

| Solid State fermentation (SSF) | Submerged liquid fermentation (SLM) |
|--------------------------------|---|
| Limited water consumed | High volume of water consumed |
| Buffered solid substrate | Easy pH control |
| Spore inoculum substrate | Easy inoculum continuous process |
| Less chances of contamination | High chances of contamination in submerged fermentation |

2.5 Local isolation fungi

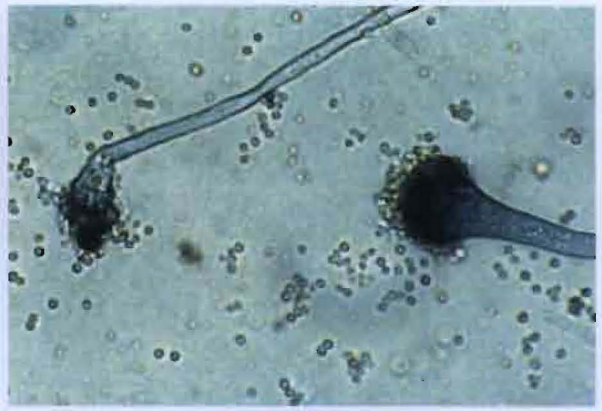
During the biovanillin production, *Aspergillus fumigatus* and *Pycnoporus cinnabarinus* or *Phanerochaete chrysosporium* was used. Stentelaire *et al.* (2000) stimulated that a two-step process was described involving the transformation of FA into vanillic acid using *Aspergillus* sp. and later converted into biovanillin by *Pycnoporus cinnabarinus* or *Phanerochaete chrysosporium*.

2.5.1 *Aspergillus fumigatus*

Generally fungi are well known agents of decomposition of organic matter and cellulosic substrate in particular (Lynd & Weimer, 2002). The genera *Aspergillus* sp. had a number of characteristics which make them ideal organisms to undergo the fermentation process. For instance, this fungus had good fermentation capabilities, high levels of protein secretion, ability to assimilate various organic substrates, suppressing the development of other microorganisms and high sporulation capacity. One of the *Aspergillus* sp. is *Aspergillus fumigatus*. This fungus was widespread in nature which it typically found in soil and decaying organic matter, such as compost heaps, where it plays an essential role in carbon and nitrogen recycling. This fungus can also use to generate the biovanillin. Samson, (1994) stated that *A. fumigatus* is a fast grower where the colony size can reach 4 cm within a week when grown on Czapek-Dox agar at 25 °C. *A. fumigatus* is a thermophilic species that can growth at temperature as high as 55 °C and survive at temperature up to 70 °C. The colour of the *A. fumigatus* colony on surface as shown in **Figure 2.5.1**, is dark green or brown fungus when it was grow on PDA medium.



(a)



(b)

Figure 2.5.1(a): 7 days growth of *A. fumigatus* F01 on PDA and Figure 2.5.1(b): Light microscopy of *A. fumigatus* sporulating structures. Retrieved from <http://mycota-crcc.mnhn.fr/site/specie.php?idE=89>.

2.5.2 *Phanerochaete chrysosporium*

Phanerochaete chrysosporium is a fungus that used in the biological treatment to degrade the lignin. It was used to allow better access to the cellulose and hemicellulose components, besides to be also considered as an effective biological detoxification alternative (Mussatto & Teixeira, 2010).

According to Martinez *et al.* (2008), *Phanerochaete chrysosporium* is the model white rot fungus because of its specialized ability to degrade the abundant aromatic polymer lignin, while leaving the white cellulose nearly untouched. *Phanerochaete chrysosporium* releases extracellular enzymes to break-up the complex three-dimensional structure of lignin into components that can be utilized by its metabolism. This *Phanerochaete chrysosporium* sustainability at moderate to higher temperatures, specifically 40 °C.

3.0 MATERIALS AND METHODS

3.1 Materials

Potato Dextrose Agar (PDA), Tween 80 solution, sago bark, sterile petri dish, oven, sieve, autoclave machine, minimal salt medium (MSM), cellulose acetate membrane (pore size 0.25 μm), 60% methanol, 40% distilled water, SP830 spectrophotometer (Metertech) , destarched rice husk (DSRH), phosphate buffer, incubator, boiling water, himac CR21G centrifuge (Hitachi), dry ferulic acid, distilled water, test tube, falcon tube, Folin-Ciocalteu reagent, and sodium carbonate.

3.2 Methods

3.2.1 Fungal strains preparation and spore calculation

Aspergillus fumigatus F01 strain was obtained from Department of Molecular Biology, University Malaysia Sarawak (UNIMAS). The strain of *A. fumigatus* was subcultured on Potato Dextrose Agar (PDA) medium for 7 days at room temperature in order to allow sufficient spore formation. Inoculum was prepared in the form of spore suspension as shown in **Figure 3.2.1.1**, by reaping with the aid of 0.01% (v/v) Tween 80 solution with standardized concentration of 1×10^6 spore/ml. The hemacytometer was prepared to calculate the spore of *A. fumigatus*. The surface of the hemacytometer and the coverslips was cleaned with ethanol. Then, 10 μl of the spore suspension was introduced into one of the square shaped wells using pipet. Later, hemacytometer was observed under the microscope to calculate the spore (*Appendix A*).

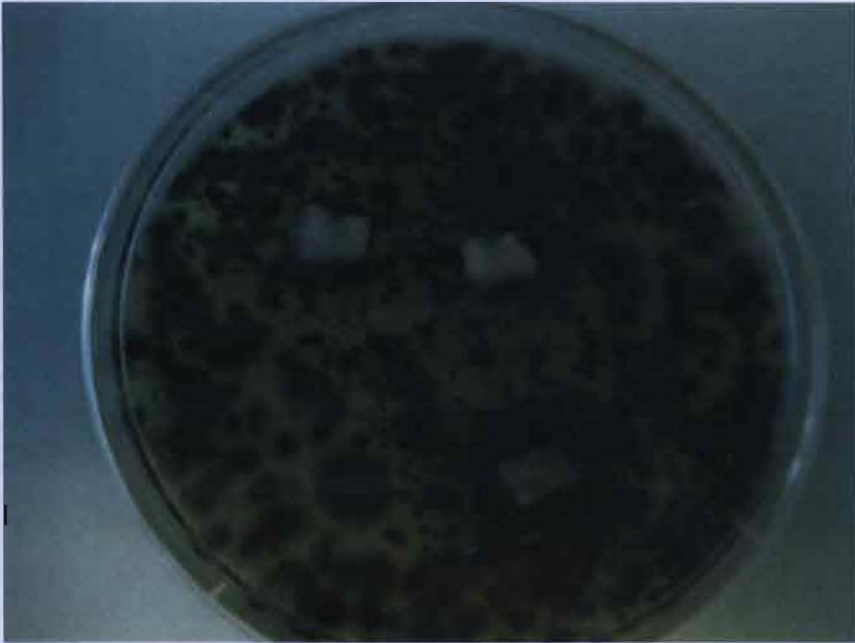


Figure 3.2.1: The subcultured of *A.fumigatus* growth on PDA for 7 days



Figure 3.2.1.1: 5 ml inoculum of 7 days harvested *A.fumigatus* F01 in Tween 80